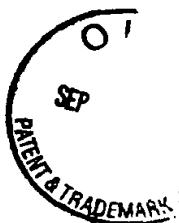


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IMMUNOGENIC CANCER PEPTIDES AND USES THEREOF

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5 This invention relates to novel general methods and compositions that provide cancer-specific or highly cancer-associated antigens useful for diagnosis and treatment of cancer.

BACKGROUND

10 The utilization of cancer-specific antigens and molecular markers in the diagnosis and treatment of malignant tumors is a goal of medical professionals. The realization of this goal has been advanced by the use of *in vivo* animal and *in-vitro* model systems in order to map out the relevant steps of a cancer-specific immune response and also the steps required for its use in cancer therapy. Methods which
15 have been reported, but the principal shortcoming preventing the implementation of these methods has been the paucity of cancer-specific or highly cancer-associated antigens and other markers of cancer in humans.

Some progress in obtaining candidates for cancer-specific or highly cancer associated antigens for cancer diagnosis and treatment includes the construction of
20 synthetic peptides, for example, for the production of antibodies specific for the peptides, where the peptides are potentially useful as markers. For example, different epitopes have been found to be associated with mucins from malignant cells, in contrast to mucins in non-malignant cells. Aberrant glycosylation has been found in some peptides from tumors.

25 Vaccines and immunotherapies using specific domains of membrane proteins have been reported to be more effective than vaccines and immunotherapy using entire glycoproteins.

At present, not enough cancer antigens or markers are available for use in implementing robust cancer diagnostic or therapeutic methods in humans. Human
30 cancer antigens and markers described to date are either inadequate or too few in number to provide useful clinical tools.

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For example, the MAGE family of antigens described by Boon *et al.* (1994) are reported to be cancer-associated antigens. Cancer-associated antigens are those expressed in greater quantity in molecules in or on, or derived from cancer cells, but are also concurrently expressed in molecules from normal cells. This duality complicates therapeutic utility of the antigens for vaccines and antibodies where positive effects are dependent upon reaching a therapeutic dose before a toxic dose level is realized. Other limitations of the MAGE antigens are that they are also intracellular cancer antigens thus greatly diminishing their utility for cancer cell targeting which is more effective for cell surface antigens. Intracellular antigens serve as poor localizing targets for immunotherapy, targeted cytotoxic therapeutic agents, cell receptor blocking agents, other cell-surface disruptive agents, and for diagnostic imaging. They are poor immunogenic targets for eliciting a measurable immune response. Their release for direct quantification is unpredictable because cancer cell disruption is required.

Cheever *et al.* (1997a,b) have described the potential diagnostic and therapeutic use of oncogenic proteins which are expressed by both cancer and normal cells. They describe using oncogenic proteins with site-specific mutations as the cancer-specific antigens. However, the oncogenic proteins cited by Cheever, designated the p21 proteins, are intracellular and thus share the drawbacks of other intracellular antigens, that is, cannot be detected on cell surfaces. Furthermore, mutated expression is not always manifested by expressed oncogenic proteins in all cancer cells, thus leaving some cells to express oncogenic proteins which are subject to self-recognition and are thus poorly immunogenic.

Cheever's other example, the erbB-2 epidermal growth factor receptor, also known as HER-2/neu, is used to support the hypothesis that breaking self-recognition offers a novel therapeutic pathway (Disis *et al.*, 1998a, b; 1999) although that method is not commonly accepted by most immunologists. The erbB-2 molecule is a transmembrane receptor with a significant extracellular portion. Its extracellular domain is commonly believed to be structurally similar for both cancer cells and normal cells. Thus, the advantages it possesses over intracellular antigen candidates is minimized because of its susceptibility to down regulation of any specific immune response on the basis of self recognition.

Use of derivatives of bombesin, an amphibian protein, was an attempt to inhibit growth of tumor cells that respond to bombesin (Knight *et al.*, 1997). Bogden and Moreau attempted to treat human cancer by administering analogs of a biologically active peptide to a patient. However, these attempts used molecules that
5 did not differentiate normal from cancer cells.

The deglycosylated mucins described by Barratt *et al.*, 1998 and Henderson *et al.*, 1998 are another example of a class of cancer-associated antigens with epitopes detectable outside of the cell. Mucins are large secreted and/or transmembrane glycoproteins with greater than 50% of their molecular weight derived from O-linked
10 carbohydrates attached to serine and threonine. Their cancer specificity depends on a greater degree of altered structure rather than on numerical over-expression. The loss or diminution of carbohydrate side chains emanating from a central core protein makes the Muc proteins more immunogenic. Finn *et al.* ascribes this immunogenicity as a result of significant altered molecular folding made possible by a release from
15 molecular rigidity conferred by the many projecting glycoside chains found in mucin molecules in non-cancerous cells. The alteration in folding creates neo-epitopes which help break immune self-recognition and also separately facilitates stimulation of a cellular immune response. Problems with the Muc antigens include insufficient diversity needed to provide wide enough antigenic coverage for many cancers, and
20 their rapid cellular release rate as a consequence of Muc antigens being secreted proteins, as opposed to functional cell membrane proteins such as receptor molecules, receptor-like molecules, or cell adhesion molecules. The latter attribute makes Muc antigens less effective therapeutic and imaging targets.

Hudziak *et al.* (1998a, b) describes the therapeutic utility of monoclonal
25 antibodies specific for the extracellular domain of the normal HER-2/neu receptor (also known as erbB-2). The basis of this therapeutic method is described as the inhibition of the cancer-proliferative function of the receptor caused by the binding of a specific monoclonal antibody to the outer domain of the receptor thereby preventing the binding of circulating epidermal growth factor and other ligands to the receptor.
30 Decreased or absent growth factor stimulation results in cancer cell death through apoptosis. This method relies on higher expression of Her-2/neu on cancer cells as compared to normal cells. Therapy is dose dependent. Sufficient blocking antibody

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must be administered so as to block enough cancer cell HER-2/neu molecules required to affect cancer cell death without causing normal cell death or normal cell toxicity. Adequate therapeutic dosing is not possible for all patients who express HER-2/neu on the their tumor cells. Some cancer patients express adequate amounts of HER-2/neu; some express low amounts; and yet others express none. Consequently, this therapeutic method works marginally, or not at all for most patients. Occasionally, when patient circumstances are appropriate, this method is capable of affecting total cancer remission. This limited result illustrates the basic soundness of a therapeutic method provided that a large repertoire of cancer-specific or cancer-associated functional targets were made available. However, more and better cancer-specific and cancer-associated antigens are needed to make these approaches clinically useful.

A method of preparing phosphorylated tumor specific peptides was reported by Calenoff (1998).

There are suggestions of expression of cancer-specific or cancer-associated molecules, as well as over-expression or under-expression of the molecules in or on cancer cells. For example, many receptor-like adhesion proteins found on the surface of cells have been described. Some of these adhesion proteins are reported to facilitate tumor migration and invasion (Zheng *et al.*, 1999; Rabinovitz *et al.*, 1995; Friedl *et al.*, 1998) or metastatic spread (Romanov *et al.*, 1999). Others are reported to facilitate essential functioning for both cancer cells and tissues and for normal cells and tissues (Ekblom *et al.*, 1998; Fleischmajer *et al.*, 1998; Bonkoff, 1998; Fujiwara *et al.*, 1998; Lohi, 1998). Blocking certain functions facilitated by receptor-like adhesion molecules is suggested to provide new therapeutic modalities for eradicating or controlling cancer (Ruoslahti *et al.*, 1997). Although various adhesion molecule isotypes are reported to be over-expressed (Damiano *et al.*, 1999; Liapis *et al.*, 1996; Begum *et al.*, 1995; Katsura *et al.*, 1998) or underexpressed (Furakawa *et al.*, 1994; Damjanovich *et al.*, 1997; Luguki *et al.*, 1999) on cancer cells as compared to normal cells, none have been described which possess the cancer-specific or highly cancer-associated structural modifications of the present invention.

SUMMARY OF THE INVENTION

The invention relates to general methods and compositions that provide cancer-specific or highly cancer associated antigens useful for cancer diagnosis and treatment. An aspect of the invention is algorithms for determining, selecting and/or constructing synthetic peptides that are candidates for producing a cancer-specific or cancer-associated immune response useful in the diagnosis and treatment of cancer.

The invention also relates peptides selected by the methods of the present invention. The peptides are preferably small, *e.g.* from 3 to about 1000 amino acids in length, and are centered around amino acids that are generally glycosylated in non-cancerous cells, and are on the cell surface, but are not glycosylated in cancer cells. More preferred lengths of the peptides are from 3-7 amino acids or 3-10, or 5-10, although peptides up to about 25 or to 1000 amino acids in length, are also within the scope of the invention. The peptides are also hydrophilic. The peptides or fragments thereof include any variation in the amino acid sequence, whether by conservative amino acid substitution, deletion, or other processes, provided that the polypeptides are in accord with the criteria of the present invention. More specifically, more than one peptide, the sequences of which are in accord with the criteria of the present invention, are preferably present to enhance the discriminatory power of the immunoassays and therapies disclosed herein. That is, a plurality of antigenic peptides forms an array (or repertoire) of molecules suitable for diagnosis and treatment of cancer.

A peptide of the present invention contains both unmodified and modified amino acids. It is recognized that the conversion of a normal to a cancerous cell type likely involves many steps. At some point, a cell (more precisely, a group of cells -- for example, a tumor) becomes distinguishable as a "cancer cell". If at that point, an amino acid differs in its state from that in non-cancerous cells, it is defined herein as "modified." Not all the cells in a cancerous tissue necessarily have the modification. For purposes of the present invention, it suffices that the modification allows some cancer cells to be distinguished from normal cells by detection of the modification or modifications.

On the external domain of proteins of cells with normal growth patterns, asparagine is the most frequent site of glycosylation, but in cancer cells the peptides of

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the present invention are missing a glycosidic complex altogether. The absence of the glycosidic complex is expected to confer a cancer-specific or highly cancer-associated immunogenicity to the altered peptide region. Deglycosylation is expected to remove steric hindrance present in non-cancerous cells, to phosphorylation or other
5 modifications of the neighboring amino acids. Removal of steric hindrance allows available phosphorylases to add phosphate groups to amino acids usually under the glycosidic umbrella. Addition of phosphate groups facilitated by deglycosylation provides an additional cancer-specific or cancer-associated molecular structure to be detected.

- 10 The immunogenic peptides of the present invention may include one or more of the constituent amino acids that are chemically modified, either in the natural state of the cancer cells, or synthetic, and the chemical modification confers upon the peptide a cancer-specific or highly cancer-associated immunogenicity or structured uniqueness that is different from, and may be independent of, the specificity or
15 association related to the altered (deglycosylated) glycosylation sites.

Following the steps outlined in Table 1, peptides suitable for the practice of the invention result in peptides with the formulas shown in Table 2.

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Table 1: Steps in Obtaining Cancer Specific of Cancer-Associated Antigenic Peptides

5	<p><u>Step 1:</u> obtain amino acid sequence of the extracellular domain of a candidate molecule <i>e.g.</i> a receptor or receptor-like molecule.</p>
10	<p><u>Step 2:</u> map hydrophilic regions of the domain by analyzing the amino acid sequence of the domain of step 1 employing the rolling sum analysis of 7 consecutive residues.</p>
15	<p><u>Step 3:</u> identify the hydrophilic regions of step 2 that are glycosylated in non-cancerous (normal) cells, but are deglycosylated in cancer cells. The deglycosylated regions of the peptide are candidates for being cancer-specific or cancer associated peptide antigens.</p>
20	
25	<p><u>Step 4:</u> look for amino acids to either side of the deglycosylated amino acids identified in step 3 that are susceptible to alteration in the absence of steric hinderance by glycoside chains.</p>
30	
35	<p><u>Step 5:</u> synthesize candidate peptides that fit the criteria obtained in steps 3 or 4 and label the peptides at one end <i>e.g.</i> with biotin.</p>
40	<p><u>Step 6:</u> use synthesized peptides as source antigens in immunoassays used to measure peptide-specific antibody in biological fluids (i.e. serum) from cancer patients and biological fluids from control subjects. Peptides which specifically complex with antibody in cancer patient fluids but not in control fluids are cancer-specific antigens. Peptides which complex with antibodies in cancer patient fluids more frequently than the complex with antibodies from control fluids from (non cancerous patient, or at least not know to be cancer patients) are designated cancer-associated antigens. Peptides which complex with antibody in both cancer patient fluids and also control fluids, or with neither, are neither cancer-specific nor cancer-associated.</p>
45	
50	

The invention is generally directed to immunogenic peptides which include a sequence of three or more amino acids, possess a net hydrophilic character, and contain at least one amino acid that is glycosylated in normal cells (generally an

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asparagine residue) but deglycosylated in cancer cells. As can be seen for the general forms in Table 2, the deglycosylated amino acid is located no further than:

1. 3 unmodified amino acids away from a fourth unmodified amino acid on either side of the deglycosylated amino acid;
2. 3 amino acids away from the most distal modified amino acid found on either side of the deglycosylated amino acid, where distal refers to a location from a deglycosylated amino acid;
3. 6 amino acids away from another deglycosylated amino acid (if there are no modified amino acids in between the two adjacent deglycosylated amino acids).

Arrays include differentiating pluralities of peptides of the present invention, to diagnose cancer.

An aspect of the invention is immunoassays employing immunogenic peptides to measure specific peptide-reactive antibodies in biological fluids, more specifically: an aspect of the invention is monoclonal antibodies and antibody-like molecules such as Fab2 and FAb fragments, known to those skilled in the art, and recombinant proteins thereof, which are specifically reactive with the immunogenic peptides of the present invention. Immunoassays employing these antibodies or antibody-like molecules of the present invention are used to measure in biological fluids, molecules containing altered peptide regions which correspond *in vivo* to the immunogenic peptides of the present invention.

Cancer imaging reagents are developed using labeled molecules of the present invention including antibodies or antibody-like molecules, directed toward cancer specific or cancer-associated peptides of the present invention. Suitable labels include radioisotopes, a paramagnetic label, and a water density label. The labels complexed with the antibodies or antibody-like molecules target cancer cells and tissues and respond to image detectors to identify the location of the cancer.

A therapeutic vaccine containing one or more immunogenic peptides of the present invention, and prepared by methods known to those skilled in vaccine development, is an aspect of the invention. Adjuvant/peptide conjugates including the immunogenic peptides coupled to molecules which facilitate enhanced immunogenicity, are used to stimulate the host immune system to facilitate the killing of cancer cells and thereafter maintain immune surveillance in case of cancer recurrence.

Vaccines created by recombinant techniques containing immunogenic peptides together with adjuvant molecular sequences which promote increased immunogenicity

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of the immunogenic peptides to stimulate the host immune system to facilitate the killing of cancer cells and thereafter maintain immune surveillance in case of cancer recurrence, are also within the scope of the invention.

DEFINITIONS

5 The term "antigen presenting cell" (APC) includes "professional antigen presenting cells" that constitutively express MHC class II molecules (e.g., B lymphocytes, monocytes, dendritic cells, Langerhans cells, and activated T cells in humans) as well as other antigen presenting cells that are capable of presenting antigen to T cells. APCs can express the appropriate combination of MHC molecules and costimulatory and/or adhesion molecules known in the art to be sufficient for presentation of antigen to T cells or can be induced or engineered to express such molecules.

10 As used herein, the term "immune response" includes T cell mediated and/or B cell mediated immune responses that are influenced by modulation of T cell costimulation. Exemplary immune responses include T cell responses, e.g., proliferation, cytokine production, and cellular cytotoxicity. In addition, the term "immune response" includes immune responses that are indirectly effected by T cell activation, e.g., antibody production (humoral responses) and activation of cytokine responsive cells, e.g., macrophages.

15 "Unmodified amino acids" are those found in the non-cancerous state -- that is, as the amino acids exist in normal cells *i.e.* non-cancerous cells. Modified amino acids are those that exist in altered states in cancerous cells.

20 The term "markers," as used herein, includes any molecule which is detectable in a biological sample and indicates the presence of another molecule of interest. Some markers are antigenic. Markers are useful because their presence is associated with a disease or condition of interest. Markers of interest herein are those whose presence is associated with cancer.

25 The single letter code for amino acids, well known to those of skill in the art, is used herein.

30 BRIEF DESCRIPTION OF THE DRAWINGS

 FIG. 1 presents the amino acid sequence (SEQ ID NO: 67) of the human epidermal growth factor receptor (EGFR); larger letters depict extracellular portions of human epidermal growth factor receptor (EGFR); the bold N denotes normally glycosylated asparagine residues on the EGFR extracellular portions; underlined amino acid sequences = hydrophilic peptide regions on extracellular portion of EGFR.

FIG. 2 presents amino acid sequence (portions of SEQ ID NO: 67) position numbers that indicate regions of cancer-specific/highly cancer-associated immunogenic peptides from the sequence shown in FIG. 1; N depicts deglycosylated asparagine in cancer-specific or highly cancer-associated immunogenic peptide regions; an underlined S, T or Y respectively depicts serine, threonine, and tyrosine amino acids which can become aberrantly phosphorylated because absent polysaccharide complexes emanating from the highlighted asparagines no longer sterically prevent various phosphorylases from approaching phosphorylatable amino acids and attaching a phosphate group; the addition of a phosphate group creates a novel immunogenic peptide region centered by the phosphorylated amino acid(s) as well as the deglycosylated asparagine(s).

FIG. 3(a) graphically indicates screening results using the EGFR peptide (portion of SEQ ID NO: 67) rNvs; the x-axis shows results for 2 groups of serum samples:

- from patients with squamous cell carcinoma (dark circles);
- samples from patients not known to have any cancer (open circles);

a dotted line shows the control (non-cancerous) population mean background +2.5 standard deviations antibody levels (serum IgG) shown on the y-axis were below the mean background + 2.5 standard deviations of the mean (+ 2.5 SD) for all samples from persons not known to have cancer, whereas 2 of 45 of the samples from persons with squamous cell carcinoma, had antibody levels above the same mean + 2.5 SD; this indicates that this peptide region of the epidermal growth factor receptor erbB-1, in altered form, likely serves as a cancer-specific immunogen or target.

FIG. 3(b) graphically indicates screening results using the EGFR peptide (portion of SEQ ID NO: 67) rNvSrgr; the x-axis shows results for 2 groups of serum samples:

- samples from patients with squamous cell carcinoma (dark circles);
- samples from patients not known to have any cancer (open circles);

antibody levels (serum IgG) shown on the y-axis) were below the mean background + 2.5 SD for the serum samples from persons not known to have any cancer, whereas 3 of 45 serum antibody levels were above the same mean +2.5 SD for the serum samples for persons with squamous cell carcinoma; although the peptide antigen used to elicit these results is structurally related to the peptide (portion of SEQ ID NO: 67) rNvsr, the serum antibody levels elicited for the peptide (portion of SEQ ID NO: 67) rNvSrgr are much higher thus indicating that adding an aberrantly phosphorylated

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extension offers a neoantigen which complexes with specific serum antibody in excess to that afforded by the (portion of SEQ ID NO: 67) rNvsr peptide alone; this, too, indicates that this peptide region of the epidermal growth factor receptor erbB-1, in a second altered form, likely serves as a cancer-specific immunogen or target.

5 FIG. 4 graphically indicates screening results using the TROP1 peptide with the amino acid sequence (SEQ ID NO: 68) aemNgSk; the x-axis shows 2 groups of results:

- a. serum samples from persons with squamous cell cancer (dark circles); and
- 10 b. serum samples from persons not known to have cancer (open circles); the y-axis shows IgG antibody levels; 6 of 45 sera from cancer patients were above the mean background level from the controls + 2.5 SD, whereas only one serum from the control population was above that level; this indicates that this peptide region of the TROP1 cell surface molecule in altered form, likely serves as a highly cancer-
- 15 associated immunogen or target; the single positive result within the control population may also be indicative of silent (clinically undetectable) cancer presence in the affected subject.

FIG. 5 graphically illustrates serum antibody levels obtained with a plurality of 4 biotinylated peptides used as test antigens; the x-axis shows 2 groups:

- 20 a. samples from persons with squamous cell cancer (dark circles);
- b. samples from persons not known to have cancer (open circles); the y-axis shows IgG antibody levels; 11 of 45 sera from cancer patients were above the control mean background level + 2.5 SD, whereas only one serum from the control population was above that level; this graph illustrates the positive summative effect of
- 25 using a sufficiently large number of non-homologous synthetic peptides corresponding to the humorally antigenic peptide regions of cancer cell receptors and/or receptor-like molecules; by having enough suitable antigenic peptides in the antigen mix of the described immunoassay method, a point is reached where enough antigenic peptides are available to provide the immunoassay with a sensitivity
- 30 approaching 100 percent while maintaining high specificity.

FIG. 6 graphically illustrates serum antibody levels obtained with 9 biotinylated peptides used as test antigens; the x-axis shows 7 groups:

- a. samples from persons with Stage I prostate cancer (dark circles);
- b. samples from persons with Stage II prostate cancer (open circles);
- 35 c. samples from persons with Stage III prostate cancer (dark squares);

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- d. samples from persons with Stage IV prostate cancer (open squares);
- e. samples from persons with benign prostatic hypertrophy (BPH), a non-malignant enlargement of the prostate (diamonds with crosses inside);
- f. samples from men not known to have cancer or BPH (open diamonds);
- 5 g. samples from women not known to have cancer (dark triangles).

The y-axis shows IgG antibody levels; 4 of 7 sera (57%) from Stage I prostate cancer patients were above the mean background level + 2.5SD; 3 of 7 sera (43%) from Stage II prostate cancer patients were above that level; 2 of 3 sera (67%) from Stage III prostate cancer patients were above that level; and 0 of 1 sera (0%) from Stage IV prostate cancer patients were above that level; whereas, only one serum (3.6%) from the BPH population and none of the normal males or females were above the threshold level; this graph also illustrates the positive summative effect of using a sufficiently large number of non-homologous synthetic peptides corresponding to the humorally antigenic peptide regions of cancer cell receptors and/or receptor-like molecules.

FIG. 7 is a diagram showing a glycosylated (CHO) amino acid (dark circle) in a peptide (chain of circles) in a non-cancerous cell and an outer membrane protein of a receptor - or receptor like molecule (OMP) with a transmembrane region (dashed line through a cell membrane (CM)) attached to an inner cell portion (small dotted circle).

FIG. 8 is a diagram of the same structure as in FIG. 7 with the exception that the glycosylated amino acid in FIG. 7 (dark circle) is now deglycosylated, as in a cancerous cell.

FIG. 9 is a diagram of the same of structure as in FIG. 8 except that one of the amino acids (shaded circles) in the peptide is phosphorylated (P), another modification in a cancer cell in addition to the deglycosylation shown in FIG. 8.

DESCRIPTION OF THE INVENTION

The invention relates to methods and compositions for obtaining cancer specific or cancer associated antigens (generally antigenic peptides) for use in diagnosis and treatment of cancer. An aspect of the invention is algorithms for determining, selecting and/or constructing antigens that are suitable for use in diagnostic tests for cancer, for producing cancer-specific or cancer-associated antibodies for use in diagnosis or treatment of cancer, and for producing immunogenic constructs for treatment of cancer. Aspects of the invention include a large repertoire

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(array) of cancer-specific and cancer-associated peptide antigens located on the surface of externally expressed cellular receptors or receptor-like molecules.

An algorithm for a peptide of the present invention directs among other things, that in the amino acid sequence, no more than 3 unmodified amino acids are located on either side of a modified amino acid (see Tables 1 and 2). Amino acid modification may include phosphorylation, conjugation of oxidized radicals (Tsimikas *et al.*, 1999; Brame *et al.*, 1999), and/or conjugation of glycosides (Shamsi *et al.*, 1998) which differ from the glycosides which are normally attached to the peptide.

The antigens of the present invention generally have the following attributes and characteristics:

- a. the antigens possess cancer-specific or cancer-associated alterations which confer antigenic and/or structural specificity upon the extracellular domains of commonly expressed receptor or receptor-like molecules;
- b. the receptors and receptor-like molecules are potentially expressed by all cancer types thereby providing broad-based antigenic diversity and significant quantitative expression for most cancers;
- c. the receptors and receptor-like molecules can serve as cell surface cancer-specific or cancer-associated antigens or as cell surface cancer markers;
- d. the receptors and receptor-like molecules are found as early as Stage I as well as in Stages II, III, and IV of cancer progression;
- f. because the receptor and receptor-like repertoire is significantly large, diminished expression among different cancers of some receptor or receptor-like molecules of the array is compensated by the standard expression or over-expression of other receptors or receptor-like molecules of the repertoire thereby providing sufficient antigenic and/or marker coverage. This varying molecular expression allows diagnostic discrimination of individual cancer types;
- g. because the receptor and receptor-like repertoire is significantly large, enough receptor or receptor-like molecules are available which either remain affixed to the outer surface of cancer cells thereby serving as ideal antigenic or marker targets for diagnosis or therapy, or are predictably released into the peripheral circulation or biological fluids which bath the cancer cells thereby serving as shed cancer antigens to be measured for diagnostic purposes.

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The antigen repertoire of this invention is different from the antigens reported by others in the following ways:

- a. the MAGE antigens of Boon (1994) expressed in cancer cells are not structurally unique compared to MAGE concomitantly expressed in normal cells. MAGE antigens are intracellularly expressed and therefore require cancer cell damage or fragmentation for reliable extracellular expression;
- b. the antigens described by Cheever (1997) possess cancer-specific structural alterations but are intracellular, or are over-expressed on the outer surface of cancer cells, but lack cancer-specific structural alterations which would confer immunogenic and/or marker specificity;
- c. the Muc antigens described by Finn (1998 a , b) possess cancer-associated structural specificity that confers antigenic and/or marker specificity but are excreted and thereby poorly retained on the cancer cell surface. The structural alterations of Muc antigens are different from the alterations described for the antigens of the present invention. The antigenic/marker sites of peptides for this invention are small and are therefore less affected by a twisting-type of conformational change but rely more on peptide denuding and upon the modification of amino acids which are normally hidden by attached glycosides. The Muc antigen repertoire is numerically insufficient to provide across the board coverage for adenocarcinomas which express Muc antigens;
- d. the antigenic site employed in the method of Hudziak (1998 a,b) to target the HER-2/neu epidermal growth factor receptor is described as being structurally similar to HER-2/neu expressed by non-cancerous cells.

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Table 2: Examples of formulas for suitable candidate cancer antigenic peptides (where u = unmodified amino acid; N = deglycosylated amino acid; M = modified amino acid and []_n symbolizes *n* number of repeats of a basic unit in brackets) are:

5	uNu
	uNuu
	Nuuu
	uNuuu
10	uuNuu
	uuNuuu
	uuuNuuu
	uNu <u>M</u> uuu
	uuNu <u>M</u> uuu
15	uuuNu <u>M</u> uuu
	uuuN <u>MM</u> uuu
	uuuNu <u>MM</u> uuu
	uuuN <u>MMM</u> uuu
	uNuu <u>M</u> uu
20	uNuu <u>M</u> uuu
	uuNuu <u>M</u> uuu
	uuuNuu <u>M</u> uuu
	uuNuu <u>M</u> uuNuu
	uuuNuu <u>M</u> uuNuuu
25	uuu <u>M</u> uuNuu <u>M</u> uuu
	[uuNuuN] _n
	[uuuNuuuN] _n
	[uuuNuuuuN] _n
	[uuuNuuuuuuN] _n
30	[uuuNuuuuuuN] _n
	[uuuNuuuuuuNu] _n
	[uuuNuuuuuuNuu] _n
	[uuuNuuuuuuNuuu] _n
35	[uu <u>M</u> uuN] _n

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Table 3: Comparison of Peptides Present in Serum From Cancer Patients to Peptide Sequences Cancer Tissue (SEQ ID NOS 1-66, respectively, in order of appearance)

	<u>Cell Surface Proteins (CSP)</u>	<u>Tested CSP Peptides</u>	<u>Prostate Cancer Serum Test Result</u>	<u>Presence of Absence of CSP in Prostate Cancer</u>
5	E-Cadherin	vkNst (SEQ ID NO: 1)	+	+
	N-Cadherin	dkNls (SEQ ID NO: 2)	+	+
	CD9	nnNNss (SEQ ID NO: 3)	-	-
10	CD38	dkNst (SEQ ID NO: 4)	-	-
	CD40	gtNkt (SEQ ID NO: 5)	+	+
	CD44	drNgt (SEQ ID NO: 6)	+	+
		NhSeg (SEQ ID NO: 7)	+	
		spNhs (SEQ ID NO: 8)	-	
	CD46	drNht (SEQ ID NO: 9)	-	-
	CD53	sdNst (SEQ ID NO: 10)	-	-
15	CD55	fcNrs (SEQ ID NO: 11)		-
	CD63	knNht (SEQ ID NO: 12)	-	-
	CD66	saNrs (SEQ ID NO: 13)	+	+
		trNdt (SEQ ID NO: 14)	-	
		skNqs (SEQ ID NO: 15)	-	
	CD82	pgNrt (SEQ ID NO: 16)	-	-
	desmoglein 1	tkNgt (SEQ ID NO: 17)	-	-
20	desmoglein 2	kiNat (SEQ ID NO: 18)	-	-
	erbB-1	daNkt (SEQ ID NO: 19)	+	+
		perNrt (SEQ ID NO: 20)	-	
		crNvs (SEQ ID NO: 21)	-	
	erbB-2	dtNrs (SEQ ID NO: 22)	+	+
	erbB-3	heNct (SEQ ID NO: 23)	-	+
	erbB-4	aeNct (SEQ ID NO: 24)	-	-
		pdNct (SEQ ID NO: 25)	-	
25	fgfr1	esNrt (SEQ ID NO: 26)	-	+
	fgfr2	ekNgs (SEQ ID NO: 27)	-	+
	fgfr4	diNss (SEQ ID NO: 28)	-	-
	hepatocyte gfr	vgNks (SEQ ID NO: 29)	-	+
	pdgfr alpha	eeNns (SEQ ID NO: 30)	-	+
30	pdgfr beta	kdNrt (SEQ ID NO: 31)	-	-
		trNvs (SEQ ID NO: 32)	-	
	ICAM 1	hkNqt (SEQ ID NO: 33)	-	+

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	integrin alpha 1	qrNit (SEQ ID NO: 34)	-	-
		seNas (SEQ ID NO: 35)	-	
	integrin alpha 2	drNhs (SEQ ID NO: 36)	-	-
	integrin alpha 3	meNkt (SEQ ID NO: 37)	+	+
		leNht (SEQ ID NO: 38)	-	
		rmNit (SEQ ID NO: 39)	-	
	integrin alpha 5	kaNts (SEQ ID NO: 40)	-	-
		lrNes (SEQ ID NO: 41)	-	
5	integrin alpha 6	raNhs (SEQ ID NO: 42)	-	+
	integrin alpha 9	qkNqt (SEQ ID NO: 43)	-	-
		kgNcs (SEQ ID NO: 44)	-	
	integrin alpha (v)	qdNkt (SEQ ID NO: 45)	+	+
		kaNtt (SEQ ID NO: 46)	-	
		teNqt (SEQ ID NO: 47)	-	
		ekNdt (SEQ ID NO: 48)	-	
	integrin beta I	nkNvt (SEQ ID NO: 49)	+	+
		vtNrs (SEQ ID NO: 50)	-	
		kNvtNrs (SEQ ID NO: 51)	-	
		keNss (SEQ ID NO: 52)	-	
		eqNct (SEQ ID NO: 53)	-	
	integrin beta 3	skNfs (SEQ ID NO: 54)	-	-
10	integrin beta 5	rcNgs (SEQ ID NO: 55)	-	-
		pdNqt (SEQ ID NO: 56)	-	
	integrin beta 6	qkNss (SEQ ID NO: 57)	-	-
		evNss (SEQ ID NO: 58)	-	
	protein-tyrosine phosphatase PCP-2	seNdt (SEQ ID NO: 59)	-	-
15	protein-tyrosine phosphatase kappa	keNdt (SEQ ID NO: 60)	-	-
		ghNes (SEQ ID NO: 61)	-	
		gdNrt (SEQ ID NO: 62)	-	
	tgfr beta type II	deNit (SEQ ID NO: 63)	+	+
		eyNts (SEQ ID NO: 64)	+	
	insulin-like gf receptor	pdNdt (SEQ ID NO: 65)	-	+
		rnNtt (SEQ ID NO: 66)	-	

Table 3 column 2 to the right is a compilation of cancer-modified peptide regions to be found on 41 receptors, receptor-like molecules, or adhesion molecules reported in the literature (CSP). This table illustrates the diversity of tissue and organ types which possess receptors, receptor-like molecules, or adhesion molecules able to present with cancer-specific or highly cancer-associated structural alterations.

Table 3 third column illustrates the positive or negative reactivity between peptides representing modified peptide regions of cancer cell surface protein molecules with antibodies in sera from prostate cancer patients. The presence or absence in prostate cancer cells of the 41 tested molecules is shown as + or - in column four. Table 3 illustrates that reactivity exists between most of the modified peptides of prostate cancer cell surface molecules but shows no reactivity with modified peptide regions of molecules not found in prostate cancer, thus illustrating the ability to serologically determine cancer type by having first mapped out the peptide antigen repertoire needed to identify each cancer. These results support feasibility of using the peptides of the present invention, in particular a plurality of peptides, for cancer diagnosis.

An aspect of the invention is monoclonal antibodies and antibody-like molecules such as Fab2 and FAb fragments, known to those skilled in the art, and recombinant proteins (Hussain *et al.*, 1996).

A cancer imaging reagent is developed using molecules including labeled antibodies or antibody-like molecules directed to antigenic peptides of the present invention. Suitable labels include a radioisotopic label for the cancer imaging reagents which, upon binding to the cells that form a cancerous tumor, highlight the presence of the tumor when scanned with a nuclear medicine scanner (Goldenberg, 1993; 1999).

Another suitable label is a paramagnetic label which, upon binding to the cells of a cancerous tumor, highlights the presence of the tumor when scanned with a nuclear magnetic resonance (NMR) scanner (To *et al.*, 1992).

Another suitable label comprises a water density label which, upon binding to the cells of a cancerous tumor, highlights the presence of the tumor when scanned with a CAT scanner.

Cancer therapeutic reagents developed using the molecules including antibodies or antibody-like molecules directed to the peptides of the present invention, have at least one of the following characteristics: they (1) bind to a cancer cell and promote lysis of that cell; (2) bind to and block the function of a receptor or receptor-like molecule on a cancer cell, thereby promoting a reduction or cessation of cancer cell growth or promoting cancer cell death; and (3) carry a radioisotope or a toxin which upon binding to a cancer cell damages or promotes cancer cell death (Goldenberg, 1993).

Examples of cancer therapeutic methods which can be formulated using a suitable cancer antigens/markers array are (see Materials and Methods for details and citations):

- a. passive immunization using constructs such as engineered antigen presenting cells and production of antigen presenting dendritic cells able to stimulate the host immune system to recognize and kill cancer cells;
- b. active immunization using cancer vaccines including recombinant fusion proteins, vaccine compositions containing adjuvants, vaccine compositions containing nucleic acid molecules, recombinant microorganisms which express cancer antigens, antigen/antibody conjugates wherein the antibody acts as a delivery vehicle for targeting the antigen onto antigen presenting cells, and heat shock protein/antigen complexes;
- c. cell lytic therapeutic antibodies, cell adhesion blocking antibodies, and growth factor receptor blocking antibodies.

Therapeutic methods using the non-phosphorylated peptide antigens of the present invention, either their amino acid sequences or the corresponding nucleic acid sequences that encode the peptides include the following:

- a. passive immunization using constructs such as engineered antigen presenting cells and production of antigen presenting dendritic cells able to stimulate the host immune system to recognize and kill cancer cells;
- b. active immunization using cancer vaccines including recombinant fusion proteins, vaccine compositions containing adjuvants, vaccine compositions containing nucleic acid molecules, recombinant microorganisms which express cancer antigens, antigen/antibody conjugates wherein the antibody acts as a delivery vehicle for targeting the antigen onto antigen presenting cells, and heat shock protein/antigen complexes.

Criteria for an antigen array suitable for passive immunotherapy specific for cancerous cells include the following:

1. each type of cancer possesses antigens such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells and are also immunogenic;
2. the cancer antigens are located on the cell surface so they are sufficiently accessible for targeting T cells;

3. the cancer antigens are present at the earliest stages of cancer progression as well as during later stages;
4. each cancer cell must have on its surface a sufficient number of specific antigens to serve as an adequate target for an effective cellular-mediated immune response;
5. the cancer antigens are retained on the surface of the cancer cells for a time sufficient for the therapeutic T cells to find their target and also to retain the bound T cells for a time sufficient to affect cancer cell death.

Criteria, for an antigen array suitable for developing effective constructs for active immunotherapy include the following:

1. each cancer type possesses antigens such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells and are also immunogenic;
2. the cancer antigens are located on the cell surface to be sufficiently accessible and thus more easily recognized by the host immune system;
3. the cancer antigens are present at the earliest stages of cancer progression as well as during later stages;
4. each cancer cell has on its surface a sufficient number of specific antigens that serve as an adequate target for a humoral and/or cellular-mediated immune response;
5. the cancer antigens are retained on the surface of the cancer cells for a time sufficient for the therapeutic effector cells, and antibodies elicited by the immunostimulatory constructs to find their target and also to retain the effector cells and antibodies for a time sufficient to affect cancer cell death.

Criteria for a marker array including an antigen suitable for developing cell-lytic therapeutic antibodies include the following:

1. each cancer type possesses markers such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells;
2. the cancer markers are available in sufficient numbers on the surface of cancer cells to provide an adequate therapeutic target at the earliest stages of cancer progression as well as during later stages;
3. the cancer markers are retained on the surface of the cancer cells for a time sufficient for the therapeutic antibodies to find their target and

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also to retain the bound antibody for a time sufficient to affect cancer cell death.

Criteria for a marker array suitable for developing growth factor receptor blocking antibodies include:

- 5 1. each cancer type possesses markers such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells;
2. the cancer markers are available in sufficient numbers on the surface of cancer cells to provide an adequate therapeutic target at the earliest
- 10 3. the cancer markers are retained on the surface of the cancer cells for a time sufficient for the growth factor receptor blocking antibodies to find their target and also to retain the bound antibody for a time sufficient to affect cancer cell death;

15 Criteria for a marker array suitable for developing cell surface adhesion blocking antibodies include:

1. each cancer type possesses receptor-like adhesion molecules which are structurally unique as compared to non-cancerous cells;
2. the adhesion molecules are available in sufficient numbers on the
- 20 surface of cancer cells to provide an adequate therapeutic target at the earliest stages of cancer progression as well as at later stages;
3. the cancer markers are retained on the surface of the cancer cells for a time sufficient for adhesion blocking antibodies to find their target and also to retain the bound antibody for a time sufficient to prevent cancer
- 25 cell attachment, migration, de-differentiation or other function essential for cancer cell survival or metastasis.

Those of skill in the art recognize that identification of Stage I cancer generally provides a 90 percent or greater cure rate through the use of currently available cancer therapies (DeVita *et al.*, 1985). Therefore, diagnostic assays for early

30 stage cancer are extremely important.

Examples of cancer diagnostic methods which can be formulated using a suitable cancer antigen/marker repertoire are: cancer-specific antibody assays, cancer-specific antigen assays, and *in-vivo* cancer imaging.

Criteria for an antigen array suitable for developing cancer-specific antibody

35 assays include:

1. each cancer type possesses antigens such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells and are also immunogenic;
2. the cancer antigens are located on the cell surface to be sufficiently accessible and thus more easily recognized by the host immune system;
3. enough cancer cells have on their surface a sufficient number of specific antigens to elicit an immune response capable of being measured at the earliest stages of cancer progression as well as at later stages and among most affected patients.

Criteria for a marker array suitable for developing cancer-specific antigen-capture immunoassays include the following:

1. each cancer type possesses markers such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells;
2. the cancer markers are predictably secreted or otherwise released into the pericellular fluids to be reliably measured;
3. enough cancer cells shed enough specific marker from within a cancerous tumor to be reliably measured at the earliest stages of the tumor's progression and during later stages of most affected patients.

Criteria for a marker array suitable for developing cancer-specific imaging reagents include the following:

1. each cancer type possesses markers such as protein, peptide, carbohydrate, or lipid molecules which are structurally unique as compared to non-cancerous cells;
2. the cancer markers are available in sufficient numbers on the surface of cancer cells to provide an adequate imaging target at the earliest stages of cancer progression as well as during later stages;
3. the cancer markers are retained on the surface of the cancer cells for a time sufficient for the imaging agents to find their target and also to retain the bound imaging agent for a time sufficient to record the presence and location of the cancer.

35

Possible outcomes for peptides screened as antigens

in serum antibody assays:

1. A positive result indicating the presence of a peptide-specific antibody in cancer patient biological fluid samples, absent evidence of antibody in samples from subjects without cancer (FIG. 3) indicates the tested peptide is a cancer-specific peptide (immunogen).

2. A significantly higher positive prevalence of a peptide-specific antibody in cancer patient biological fluid samples as compared to samples from subjects without cancer (FIG. 4) indicates either that the tested peptide is cancer specific and that the few control positives have asymptomatic cancer or that the peptide serves as a highly cancer-associated antigen.

3. No difference in positive antibody levels between cancer patients and subjects without cancer. Biotinylated peptides producing these results are neither cancer specific nor highly cancer-associated.

EXAMPLES

The following examples illustrate embodiments of the invention.

Example 1: Use of the EGFR peptide on serum from cancer patients and controls.

Using immunoassay 2 (see Materials and Methods) the following results were obtained.

FIG. 3(a) graphically indicates screening results using the EGFR peptide (portion of SEQ ID NO: 67) rNvs; the x-axis shows results for 2 groups of serum samples:

- a. from patients with squamous cell carcinoma;
- b. samples from patients not known to have any cancer; antibody levels (serum IgG) were below the mean background plus 4 standard deviations of the mean (+ 2.5 SD) for all samples from persons not known to have cancer, whereas 2 of 45 of the samples from persons with squamous cell carcinoma, had antibody levels above the mean + 4SD.

FIG. 3(b) graphically indicates screening results using the EGFR peptide (portion of SEQ ID NO: 67) rNvSrgr; the x-axis shows results for 2 groups of serum samples:

- a. samples from patients with squamous cell carcinoma;

- b. samples from patients not known to have any cancer; antibody levels (serum IgG) were below the median background plus 2.5 SD for the serum samples from persons not known to have any cancer, whereas 3 of 45 serum levels were above the median + 2.5 SD for the serum samples for persons with squamous cell carcinoma.

Example 2: Use of the TROP1 peptide on serum samples from cancer patients compared to controls.

FIG. 4 graphically indicates screening results using the TROP1 peptide with the amino acid sequence (portion of SEQ ID NO: 68) emNgSk; the x-axis shows 2 groups of results:

- a. serum samples from persons with squamous cell cancer; and
- b. serum samples from persons not known to have cancer; the y-axis shows IgG antibody levels; 6 of 45 serum from cancer patients were above the median background level + 2.5 SD, whereas only one serum from the control population was above that level.

MATERIALS AND METHODS

A Method for Selecting Cancer-Specific or Highly Cancer-Associated Immunogenic Peptides and/or Markers

The identification and validation (or confirmation) of cancer-specific and cancer-associated antigenic peptide regions and/or marker peptide regions found on the extracellular domain of receptors or receptor-like molecules is performed through the use of a algorithms such as the following:.

First, the amino acid sequence of the extracellular domain of a receptor or receptor-like molecules is obtained. For example, the human epidermal growth factor receptor (EGFR), erbB-1, as illustrated in FIG. 1.

Second, the amino acid sequence is analyzed employing rolling sum analysis of 7 consecutive residues (Hopp *et al.*, 1981; Parker *et al.*, 1986; Fauchere *et al.*, 1983; Taragu *et al.*, 1990) in order to map out peptide regions which are hydrophilic and therefore apt to be expressed on the outer surface of the protein. For example, the hydrophilic regions of the EGFR outer domain are underlined in FIG. 1.

Third, hydrophilic peptide regions containing amino acids which are normally glycosylated are identified. These amino acids, illustrated by a bold capital letter N in FIG. 1, are apt to be totally (or partially) deglycosylated in cancer cells. The absence or truncation of the glycoside chain results in peptide structures which are structurally

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distinct for cancer cell proteins. The distinctly structured peptide regions can serve as a tumor-specific antigenic site if this alteration is not expressed in normal cells, or can serve as a cancer-associated antigen by cancer cells. The ability of a peptide to serve as an antigen depends on the host's immune system being able to process and recognize the peptide as an antigen. The processing and recognition of an antigen is dependent on individual MHC genotypes. If an altered peptide cannot serve as a cancer-specific or cancer-associated antigen, it may still be useful as a molecular marker of cancer on the basis of its cancer-specific or cancer-associated molecular alteration.

10 Fourth, the deglycosylated peptide regions are evaluated for the inclusive presence of amino acids that are susceptible to alteration in the absence of glycoside chains which normally would sterically restrict the contact of enzyme or other agents with the amino acids susceptible to molecular modification. The amino acid modification confers a second order alteration on the affected peptide which can result in a new and distinct peptide structure with specific antigenic and/or marker properties. Examples of such an amino acid modification include aberrant phosphorylation of serine, threonine, and tyrosine residues, malondialdehyde (MDA) modification of lysine residues, aberrant glycosylation of arginine residues, and the like.

20 Fifth, candidate peptides which fit the criteria of the cancer-modified peptide regions described in Steps 1 through 4 are synthesized and biotin labels are attached at either end of each peptide.

25 Sixth, employing the immunoassays disclosed herein, each biotinylated peptide is screened as an antigen against sera or other relevant biological fluids containing antibodies taken from one of the following groups: cancer patients, patients with benign lesions or inflammatory conditions, and healthy subjects. Peptides can also be screened using tumor-infiltrating lymphocytes or peripheral blood-born lymphocytes from cancer patients. Candidate peptides we consider cancer-specific or cancer-associated depend on whether they fit the following definitions.

Potentially useful peptides prepared in accord with steps 1-5 preferably possess the following attributes:

35 a. contain no more than 3 unmodified amino acids attached on either side of a deglycosylated amino acid or a modified amino acid. Peptides containing both deglycosylated amino acids and modified amino acids have no more than 2 unmodified amino acids between the deglycosylated amino acids and the modified

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amino acids. Peptides with more unmodified amino acids on either side of a deglycosylated amino acid become antigenically less differentiating for cancer as the respective unmodified amino acid numbers increase. The EGFR peptide (portion of SEQ ID NO: 67) daNk_g in FIG. 2 represents a peptide suitable for the practice of the invention, with a single deglycosylated amino acid at its central portion. The EGFR peptide (portion of SEQ ID NO: 67) daNk_Tg_lk in FIG. 2 represents a suitable peptide with both a modified amino acid and a deglycosylated amino acid in its central portion.

- b. a plurality of deglycosylated amino acids and modified amino acids providing that the modified amino acids proximal to a deglycosylated amino acid are no further than the third amino acid position from the nearest deglycosylated amino acid and that 2 or more deglycosylated amino acids in a peptide are connected by no more than 6 unmodified amino acids.

Synthesis of Deglycosylated Peptides and Phosphorylated, Modified Forms

- Methods of synthesis of peptides and their corresponding, encoding nucleic acid molecules are well known in the art and can be obtained commercially from U.S. companies such as the American peptide Company (Sunnyvale, CA 94086) and Commonwealth Biotechnologies, Inc. (Richmond, VA 23235).

Immunoassay Method 1: Used to Detect Serum IgA, IgD, IgE, IgG, and IgM

- Antibodies Specific for Individual Peptide Antigens**

Materials:

Neutravidin coated microtiter plates manufactured as per Example 4.

- Wash Buffer: 20 mM Tris-HCl + 150 mM NaCl + 0.05% Triton X405 + 0.2 mg/mL thimerosal, pH 7.4.

Biotinylated peptide solution containing 1.5 g/mL peptide in 20 mM Tris-HCl + 600 mM NaCl + 30 mg/mL polyethylene glycol 4000 (PEG-4000, Mallinckrodt Chemical H273-61) + 0.05% Triton X405 + 0.2 mg/mL thimerosal, pH 7.4.

- Anti-IgA/alkaline phosphatase (Kirkegaard and Perry 075-1001) + anti-IgG/alkaline

phosphatase (Kirkegaard and Perry 075-1002) solution: 0.3 g/mL of each conjugate in solution containing 20 mM Tris-HCl + 600 mM NaCl

- + 30 mg/mL polyethylene glycol 4000 (PEG-4000, Mallinckrodt Chemical H273-61) + 0.05% Triton X405 + 0.2 mg/mL thimerosal, pH 7.4.

4-methylumbelliferyl phosphate (4-MUP) fluorescing substrate solution:

25.2 mg 4-MUP (Sigma M-8883)/mL solution containing 180 mM

2-amino-2-methyl-1-propanol + 123 mM magnesium chloride, pH 9.5.

Fluorolite 1000 microtiter plate fluorometer (Dynatech) with excitation

5 set at 365 nm and emission at 450nm.

Procedure:

- 1) Adsorb neutravidin (NA) reactive antibodies and biotin reactive antibodies from serum samples by adding one neutravidin-conjugated paper disc to every 25 mL of serum and 1 biotin-conjugated paper disc to every 200 mL serum. Allow disc/serum mixture to incubate for 26 to 18 hours at room temperature, under gentle agitation.
- 2) Mix 75 mL of adsorbed serum together with 75 mL of peptide solution.
- 3) Vortex mixture and let incubate at room temperature for 40 minutes.
- 15 4) Aspirate well contents and wash microtiter wells of neutravidin plate (275mL wash buffer/well) x6.
- 5) Add 100mL biotinylated peptide/serum solution to corresponding well and incubate for 3.5 minutes.
- 6) Aspirate well contents and wash microtiter wells (275mL wash buffer/well) x6.
- 20 7) Add 100mL anti-IgA/ alkaline phosphatase + anti-IgG/alkaline phosphatase conjugate solution and incubate for 40 minutes.
- 8) Aspirate well contents and wash microtiter wells (275mL wash buffer/well) x6.
- 25 9) Add 100mL 4-MUP substrate solution.
- 10) Read derived fluorescence using microtiter plate fluorometer at 5, 10, 20, 30, and 60 minutes.

Immunoassay Method 2: Used to Detect Serum IgA, IgD, IgE, IgG, and IgM Antibodies Specific for Individual Peptide Antigens.

30

Materials :

NeutrAvidin^a conjugated paper disc, 6 mm.

Serum diluent: 10 mM sodium phosphate, pH 7.20, with 150 mM sodium chloride, and 0.20 mg/mL sodium azide.

35

NeutrAvidin^a coated white microtiter plate, stored in 10 mM Tris-HCl, pH 7.50, containing 600 mM sodium chloride and 0.2 mg/mL thimerosal.

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Plate blocking solution: 10 mM sodium phosphate, pH 7.20, containing 150 mM sodium chloride, 100 mg/mL Triton X-405, and 0.2 mg/mL thimerosal.

Plate wash buffer: 20 mM Tris chloride, pH 7.4, containing 150 mM sodium chloride, 0.5 mg/mL Triton X-405 and 0.2 mg/mL thimerosal.

- 5 Peptide solution: 0.06 μ g/mL peptide dissolved in 20 mM Tris chloride, pH 7.4, containing 600 mM sodium chloride, 30 mg/mL polyethylene glycol 4000, 1mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid, 0.5 mg/mL Triton X-405 and 0.2 mg/mL thimerosal.

- 10 Control peptide solution: 0.013 μ g/mL control peptide dissolved in 20 mM Tris chloride, pH 7.4, containing 600 mM sodium chloride, 30 mg/mL polyethylene glycol 4000, 1mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid, 0.5 mg/mL Triton X-405 and 0.2 mg/mL thimerosal.

- 15 Conjugate solution: 0.100 μ g/mL alkaline phosphatase conjugated polyclonal goat anti human IgG dissolved in 20 mM Tris-HCl, pH 7.40, with 600 mM sodium chloride, 30.0 mg/mL PEG-4000, 3.0 mg/mL BSA, 0.5 mg/mL Triton X-405 and 0.20 mg/mL thimerosal.

- 20 Substrate solution: 25.2 μ g/mL 4-methylumbelliferyl phosphate dissolved in 180 mM 2-amino-2-methyl-1-propanol, pH 9.50, containing 123 μ M magnesium chloride.

Serum preparation:

1. Add 100 μ L serum to 15 NeutrAvidin^a coated paper discs in a suitably sized test tube.
2. Incubate with gentle mixing at ambient temperature for 16-20 hours.
- 25 3. Add 7.900 mL of serum diluent and mix gently for 30 minutes.
4. Vortex the tube gently to completely release the serum from the discs.
5. Remove the treated serum from the discs and transfer it to a suitable storage tube.
6. Store the treated serum at 4 EC.

30 Assay procedure:

1. Two days before assay, aspirate the storage solution from the NeutrAvidin^a coated white microtiter plate and add 200 μ L plate blocking solution to each well.
2. Cover the plate and incubate at ambient temperature for 16-20 hours.
- 35 3. One day before assay. wash the blocked plate three times with plate wash buffer, approximately 275 μ L per well per wash. Aspirate the final wash and add 100 μ L peptide solution or 100 μ L control peptide solution to the appropriate wells of the plate.

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4. Cover the plate and incubate with gentle mixing at ambient temperature for 16-20 hours.
5. Day of assay, wash the blocked plate three times with plate wash buffer, approximately 275 μ L per well per wash. Aspirate the final wash and add 100 μ L treated serum to the appropriate wells of the plate.
6. Cover the plate and incubate at 25 EC for 2 hours.
7. Wash the blocked plate six times with plate wash buffer, approximately 275 μ L per well per wash. Aspirate the final wash and add 100 μ L conjugate solution to each assay well.
8. Cover the plate and incubate at 25 EC for 1.5 hours.
9. Wash the blocked plate six times with plate wash buffer, approximately 275 μ L per well per wash. Aspirate the final wash and add 100 μ L substrate solution to each assay well.
10. Read the plate at 30 and 60 minutes in a fluorescence microtiter plate reader set at 365 nm excitation and 450 nm emission.

Biotinylation of Human Serum Albumin

Materials:

Human Serum Albumin: Sigma A 8763

Sulfosuccinimidyl 6-(biotinamido) Hexanoate: Pierce 21335

Tris base: Sigma T 1503

20 mM sodium phosphate, pH 7.2

100 mM sodium hydroxide solution

Procedure:

Human serum albumin is dissolved in phosphate buffer at a concentration of approximately 40 mg/mL. The protein concentration of the solution is determined by absorbance at 280 nm (1 mg/mL = OD280 of 0.58) or by the Lowry method.

Immediately prior to biotinylation, the pH of the albumin solution is adjusted to 8.5 by the addition of sodium hydroxide. Succinimidyl biotin is then added at a molar ratio of 50 : 1 (422 mg succinimidyl biotin per mg albumin). The reaction mixture is vortexed thoroughly and then mixed gently for 45 minutes at ambient temperature.

Reaction byproducts and unreacted biotin are removed by extensive dialysis against phosphate buffer. The biotinylated human serum albumin is stored at 4 C.

Preparation of Covalent Ready Cyanogen Bromide (CNBr) Activated Paper Discs

Materials:

Paper discs: Schleicher and Schuell 53870

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CNBr solution: 20 gm CNBr (Sigma C6388) + 600 mL distilled water

1M NaOH

0.05M NaHCO₃

25%, 50%, 75%, and 100% acetone

5 Distilled water

Dessicant packets: Sigma S8394

Zip lock plastic bags

Procedure:

10 The following procedure is performed under a hooded, well ventilated environment. 20 gm paper discs are swelled in 200 mL distilled water at room temperature. Swelled paper discs are then added to 600 mL of CNBr solution while stirring. Bring up the pH of the stirring mixture to 10.5 and maintain at pH 10.5 until 100 mL of 1M NaOH have been used up. Aspirate the resulting liquid and wash discs with 500 mL of NaHCO₃ buffer for 2 minutes at room temperature. Repeat wash step
15 X12. Rinse discs twice with 500 mL distilled water. Rinse discs twice with 500 mL 25% acetone. Rinse discs twice with 500 mL 50% acetone. Rinse discs twice with 500 mL 75% acetone. Rinse discs twice with 500 mL 100% acetone. Aspirate last acetone wash solution and allow discs to dry under a running fume hood at room temperature. Store dried CNBr activated paper discs in zip lock plastic bags
20 containing dessicant packettes.

Preparation of Neutravidin Conjugated Paper Discs and Biotinylated Human Serum Albumin Conjugated Paper Discs

Materials:

25 Biotinylated human serum albumin: Prepared by method of Example 1

Neutravidin: Pierce 31000

CNBr-activated paper discs: Prepared by method of Example 2

Modified Coca's buffer: 0.05M NaHCO₃ +0.15M NaCl. PH 7.2

0.05M ethanolamine solution

30 0.2M sodium acetate buffer, pH 4.0.

Paper disc incubation buffer: 0.05M sodium phosphate + 0.15M NaCl +
0.05%NaN₃

+ 0.5% Tween20

35 Procedure:

A 2.5 mg/mL solution of neutravidin is prepared in modified Coca's buffer. A 2.5 mg/mL solution of biotinylated human serum albumin is prepared in modified Coca's buffer. 50 CNBr-activated discs are added to each mL of protein solution. Each protein/disc mixture is agitated for 16 to 18 hours at room temperature. Each

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solution surrounding the respective paper discs is aspirated and each set of discs are washed X3 with modified Coca's buffer. The washed discs are immersed in 0.05M ethanolamine solution and agitated for 3 hours in order to block any unreacted CNBr sites. Each set of paper discs is then washed X3 with the sodium acetate buffer.

- 5 During the third step, the paper discs are incubated in the sodium acetate buffer for 30 minutes under gentle agitation. Each set of paper discs is then washed X4 in Coca's buffer and then stored in the paper disc incubation solution at 4 C.

Preparation of Neutravidin Coated Microtiter Plates

Materials:

- 10 Amino Polystyrene Microtiter Plates (White): Nunc 453686 or the equivalent
Neutravidin: Pierce 31000
Disuccinimidyl suberate (DSS): Pierce 21555
Dimethyl sulfoxide (DMSO): Burdick and Jackson 081-1
20 mM sodium phosphate, pH 5.50
15 50 mM sodium carbonate, pH 9.6
PBS with sodium azide

Procedure:

- 20 Prepare a volume of neutravidin appropriate for the number of plates to be coated. The coating solution contains 20 mg/mL neutravidin in 20 mM sodium phosphate, pH 5.50.

Prepare a suitable volume of DSS, 1.22 mg/mL, in dry DMSO. This solution must be used within 2 hours of preparation.

- 25 For each plate to be coated, add 60 mL DSS solution to each well followed by 60 mL of 50 mM sodium carbonate, pH 9.6. Incubate this mixture in the wells for 36 minutes at ambient temperature. Aspirate the wells and wash twice with deionized water. Immediately add 100 mL neutravidin solution. Cover the plate and incubate at ambient temperature for 16-18 hours.

- 30 Aspirate the coating solution and add approximately 280 mL PBS with azide to each well. Seal the plate with a foil cover. Store the coated plates at about 4 C.

Vaccination Methods

- 35 Methods for preparing and administering a vaccine using a peptide as an epitope have been reported. For example, Gilewski *et al.* (2000) used a MUC1 peptide with a keyhole limpet hemocyanin (KLM) in a conjugate to determine whether an immune response could be generated against the MUC1 peptide that would also bind with tumor cells. An immunogenic response was reported.

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Passive Immunization Constructs That the Host Immune System Recognizes and Kills Cancer Cells

1. ENGINEERED ANTIGEN PRESENTING CELLS

5 See the "Detailed Description of the Invention" from U.S. Pat. No. 5,851,320, incorporated by reference.

2. DENDRITIC CELLS

See the "Detailed Description of the Invention" and "Examples 1-7" from U.S. Pat. No. 5,871,156 and the "Detailed Description of the Invention" and "Examples 1-5" from U.S. Pat. No. 6,080,409, incorporated by reference.

Active Immunization Using Cancer Vaccines

1. RECOMBINANT FUSION PROTEINS

See the "Summary of the Invention" from U.S. Pat. No. 4,399,216 and U.S. Pat. Nos. 6,106,829 and 5,616,477 incorporated by reference.

15 2. ADJUVANTS

See U.S. Pat. Nos. 5,750,110; 5,876,966; 5,876,735; 6,013,268 and 6,080,399, incorporated by reference.

3. NUCLEIC ACID MOLECULES

See U.S. Pat. Nos. 5,593,972; 5,817,637; 5,830,876; 6,063,384; 6,077,663; 20 5,981,505 and 5,942,235 incorporated by reference.

4. RECOMBINANT MICROORGANISMS WHICH EXPRESS
CANCER ANTIGENS

See U.S. Pat. No. 6,051,237 incorporated by reference.

25 5. ANTIBODY DELIVERS ANTIGEN TO ANTIGEN PRESENTING
CELLS

See U.S. Pat. 5,194,254 incorporated by reference.

6. HEAT SHOCK PROTEIN/ANTIGEN COMPLEXES

30 See U.S. Pat. Nos. 5,837,251; 5,981,706; 5,985,270; 5,997,873; 6,030,618 and 6,136,315 incorporated by reference.

7. CELL LYTIC THERAPEUTIC ANTIBODIES

See the section entitled "Therapy" in U.S. Pat. Nos. 4,585,742 incorporated by reference.

35 8. CELL ADHESION BLOCKING ANTIBODIES SUCH AS
INTERGRIN ANTAGONISTS

(See Kerr *et al.*, 2000).

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See U.S. Pat. No. 5,772,997 incorporated by reference.

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Bryden *et al.*, 1999;

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Fudge *et al.*, 1994;

Grasso *et al.*, 1997;

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Kramer *et al.*, 1995;Rokhlin *et al.*, 1997;

Takahashi *et al.*, 1998;

Tozawa, 1996;

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Tron *et. al.*, 1999;

Watanabe *et al.*, 1999; and

Zheng *et al.*, 2000.

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companies. Licenses are available for microchip technologies through Argonne National Laboratory.

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